COMPARISON OF CHIRAL METHAMPHETAMINE SEPARATIONS IN URINE

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Abstract:

Methamphetamine (meth) and amphetamine are commonly abused central nervous system (CNS) stimulants that are frequently screened for in many forensic and clinical toxicology labs. Amphetamine is present as the metabolite of methamphetamine, but also maybe be taken directly. These drugs exist in two chiral forms, d- and l-, called enantiomers. d-Meth is physiologically more reactive in the CNS, affecting the dopamine system and making it more likely to be abused. l-Meth is commonly found in nasal decongestants, as it has more peripheral nervous system (PNS) effects and less CNS stimulant effects than d-meth. For these reasons, d-meth is commonly abused and obtained illegally, while l-meth may be legally obtained in over the counter (OTC) nasal decongestant products.

Most drug testing laboratories used liquid chromatography with tandem mass spectrometry (LC-MS/MS) to confirm the presence of methamphetamine and amphetamine, but this doesn't typically differentiate between the enantiomers. As there is utility in understanding whether the methamphetamine in a urine specimen is from a licit or illicit source, it would be useful to develop screening methods that can differentiate between the d- and l- enantiomers in forensic and clinical toxicology labs.

In this research, a simple and cost-effective LC-MS/MS chiral separation method was developed to separate d- and l-enantiomers of methamphetamine and amphetamine. Separation methods using a chiral column with either solid phase extraction or dilute and shoot specimen preparation were developed and successfully applied to research specimens. The differentiation of enantiomers using an achiral column and a pre-column derivatization with Marfey's reagent was also researched, but the derivatives were challenging to detect in the LC-MS/MS. The successful chiral column extraction methods were applied to five (5) anonymized urine samples in a single-blind test, four negatives and one positive for methamphetamine, and it was possible to identify both d-methamphetamine and d-amphetamine.

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CHAPTER I

I. INTRODUCTION

The great opioid epidemic has gained significant national attention, with the number of associated deaths soaring to over 70,000 in 2015.[1] However, another drug quietly hangs back in the shadows of the opioid problem: methamphetamine. In 2015, nearly 4,900 methamphetamine users died from an overdose in the United States.[1] This represents a 35 % increase of meth-related deaths in just one year. In 2014, the number of meth-related deaths was 3,728, which is more than double the 1,388 methamphetamine deaths in 2013.[2-3] In Oklahoma, 271 meth-related deaths occurred in 2015; that number increased to 328 in 2016.[2]

Methamphetamine, or meth, and amphetamine are drugs that are commonly encountered and screened for in many forensic and clinical toxicology labs. These drugs are chiral compounds, or a pair of compounds that are non-superimposable mirror images of each other.[4] Both meth and amphetamine have two optical forms, d- and l-, called enantiomers. The structures of the d- and l-enantiomers of amphetamine and meth are shown in Figures 1 and 2, respectively.[5]

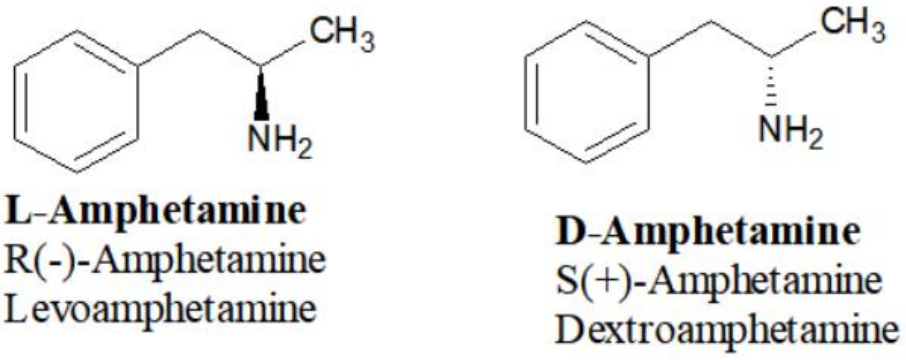

Figure 1: Structures of d- and l-amphetamine enantiomers[5]

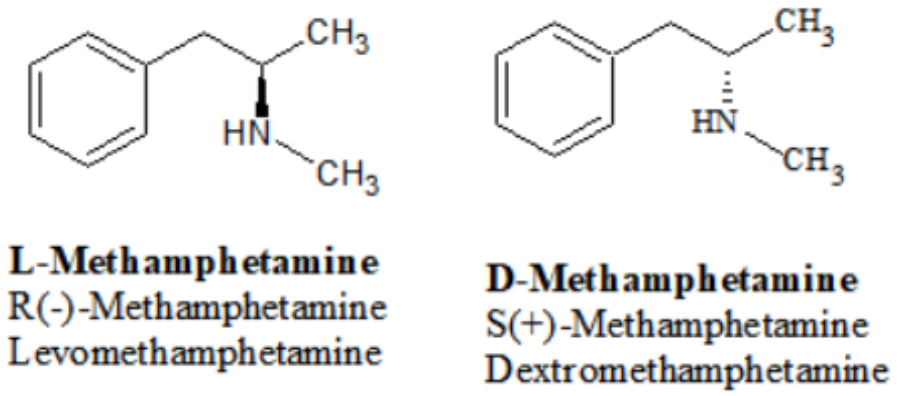

Figure 2: Structures of d- and l-meth enantiomers[5]

As seen in the above figures, enantiomers are compounds that have the same physical and chemical properties, but the body will react differently depending on the enantiomeric form.[4] The d-form of meth is more effective in the central nervous system, where it causes the release of dopamine, as well as blocking its reuptake, and is five times more physiologically reactive than the l-form.[4] This is why illicit meth that is purchased on the street will more often contain the d-form. The l-form of meth is the less reactive form of the two forms and is the active ingredient in over-the-counter nasal decongestant medicines, like Vicks VapoInhaler™.

The separation and quantification of meth and amphetamine enantiomers is not a new problem in forensic and clinical toxicology labs. Many labs will employ analytical techniques that

will detect the presence or absence of meth and/or amphetamine but will not distinguish between either enantiomer for either drug. These techniques usually only identify whether a screened sample is positive or negative for the presence of meth and/or amphetamine. If an analyzed sample is positive for meth and/or amphetamine, labs may be requested to further test the sample to identify which isomer is present to differentiate illicit use from legal or licit use.

In cases where a lab does not have a chiral separation method in place, the sample will need to be sent out to an off-site lab for the chiral analysis. However, the process of sending these positive samples to another lab for the separation analysis can get quite expensive. For these reasons, toxicology labs may need to develop techniques for the enantiomeric separation of meth and amphetamine on-site.

The main separation techniques currently employed in forensic and clinical toxicology labs involve gas chromatography (GC) and liquid chromatography (LC) coupled with mass spectrometry (MS).[4,6-8] GC-MS methods often require the addition of a chiral derivatization reagent, that is added to a sample to modify the analyte to make it more stable for analysis. The reagents are added to the enantiomers during sample preparation to form diastereomers prior to analysis.[4,6,9-10]

Diastereomers are a pair of non-super imposable mirror images of a compound that are formed during sample preparation for analysis of meth or amphetamine enantiomers. The chiral derivatization reagent must be a detector-sensitive compound for the type of analytical technique to be used in order to be effective.[9] By employing derivatization of a compound, the sensitivity and selectivity of the chemically modified sample will be increased.[10] Examples of chiral derivatization reagents include: 1-(9-fluorenyl)ethyl chloroformate (l-FLEC)[4], 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent)[4,9], 9-fluorenylmethyl chloroformate-L-proline (FMOC-L-Pro)[4], and *o*-phthalaldehyde (OPA).[4,9]

However, additions of chiral reagents can affect the overall percentages of meth and amphetamine enantiomers in samples of suspected abusers. GC-MS not only causes racemization, but also lengthens the sample preparation time prior to analysis.[6] Racemization is a chemical process where a drug will undergo a reaction to result in an equal mixture of each possible enantiomer. Racemization occurs, for example, when a sample of pure d-meth undergoes derivatization and a mixture of both d- and l-forms of meth are present in the sample.

The combination of LC combined with tandem mass spectrometry detectors (LC-MS/MS) is commonly employed in forensic and clinical toxicology labs due to its ability to detect very low amounts of drugs and substances in samples. LC-MS/MS has many advantages when compared to GC-MS for separation of enantiomers. LC-MS/MS requires less sample preparation time prior to analysis. This technique can also lead to more accurate results because racemization of enantiomers is not as likely to occur during the analytical procedure since derivatization is not employed.[6]

The existing literature shows three main methods that are employed for the separation of drug enantiomers: use of a chiral selector that acts as an additive in the mobile phase, use of a chiral column, or the addition of a chiral derivatization reagent to a sample to form diastereomers for analysis on an achiral column.[4] There are many published LC methods using chiral columns to separate meth and amphetamine enantiomers.[5-6,11-12]

A chiral column eliminates the need for pre-column derivatization of a sample because enantiomers will react differently with the internal environment of the column for separation of the enantiomers to occur.[8] The Astec Chirobiotic™ column has been established to effectively separate and quantify both meth and amphetamine enantiomers in common forensic and clinical toxicology samples, including blood, urine, and hair.[5,7,12-13] However, a major disadvantage with chiral columns is that they are often very expensive. Many forensic and clinical toxicology labs

may have strict budgets or do not have funds to purchase these columns, so labs need to find less expensive options to separate enantiomers.

A more viable option for enantiomeric separation is for labs to add a chiral derivatization reagent to the sample during the pre-column derivatization process, which is a more cost-effective option when compared to the chiral column. Marfey's reagent is one such chiral derivatization reagent that has been proven to be effective in the chiral separation of meth and amphetamine enantiomers.[4,7,14-15]

The analysis of specific enantiomers can provide law enforcement, forensic and clinical toxicology labs with useful information, such as to the location from which meth and/or amphetamine end products originated. Analysis of a drug's enantiomers can also provide helpful information as to what the identity of the starting materials that were used for a specific drug production-method were.[16] Determination of the enantiomers may also provide law enforcement information about the manufacturing process and the precursor sources used to make the end products, as pseudoephedrine results in d-meth and phenylacetone results in a racemic mixture of d- and l-meth.[4]

The purpose of this research was to develop a simple and cost-effective chiral separation method to separate d- and l-meth and amphetamine in urine samples using LC-MS/MS. Two separation methods using an Astec Chirobiotic chiral column were developed, as well as an additional method that attempted to use Marfey's chiral derivatization reagent on an achiral column.

As a result of this research, forensic and clinical toxicology labs will be able to determine the appropriate separation analyses of meth and amphetamine enantiomers. The more effective chiral separation method may enable those labs to save money by not having to send out positive

meth and/or amphetamine urine samples to an off-site lab for the enantiomeric separation analysis.

CHAPTER II

II. LITERATURE REVIEW

2.1. Overview

Methamphetamine, or meth, and amphetamine are central nervous system (CNS) stimulants that are classified as phenethylamine compounds. Other drugs in this class include 3,4-methylenedioxymethamphetamine (MDMA), 3,4-Methylenedioxy-N-ethylamphetamine (MDEA), 3,4-methylenedioxyamphetamine (MDA), 4-hydroxy-3-methoxyamphetamine (HMA), ephedrine (EPH), and pseudoephedrine (PSE).[7-8,17] Meth and amphetamine are highly abused drugs that can either be inhaled, injected, ingested, or snorted.

These highly abused drugs have some therapeutic uses. Both meth and amphetamine are used in the treatment of narcolepsy, attention deficit disorder (ADD) and attention deficit hyperactivity disorder (ADHD), obesity, and depression.[7,16-17] Meth can also be found in the prescription drug Desoxyn, which is used to treat asthma.[7] Both meth and amphetamine are chiral compounds that have two optical forms, d- and l-, called enantiomers. These can be separated using chiral columns or with a chiral derivatization reagent that forms diastereomers, which are compounds that can easily be separated, prior to analysis.

A review of the literature demonstrates that meth and amphetamine are complex drugs with a long history of abuse. There are many published studies on the separation and

quantification of d- and l-enantiomers of meth and amphetamine using chiral columns and pre-column derivatization with chiral derivatization reagents in blood, urine, plasma, oral fluid, and hair.[13,18] However, a comparison study of the separation of d- and l-meth and amphetamine enantiomers using two different chiral separation methods in urine samples is still lacking.

The goal of this chapter is to provide the reader with an overview of meth and amphetamine. The history, illegal production methods of meth and amphetamine, the definition and importance enantiomers will be discussed. Analytical methods and extraction methods that are commonly used in forensic and clinical toxicology labs to separate and quantify drug enantiomers will also be discussed.

2.2. History of Methamphetamine and Amphetamine

Meth was first created in Japan in 1893,[7] while amphetamine first appeared in 1877.[17] The use of meth and amphetamine became popular during World War II, when the military used amphetamine as stimulants to keep personnel awake and alert for prolonged combat periods.[16,18] It was also common for bomber pilots and their crews to frequently take amphetamine pills to keep themselves alert on long bombing missions.[19]

During the Gulf War, d-amphetamine pills were given to pilots to combat fatigue during long flights.[16] Amphetamine was also given to helicopter pilots during periods of sleep deprivation to improve their alertness.[16] Military personnel used meth to increase a soldier's endurance and suppress appetite during combat.[7]

After the war the use and abuse of both meth and amphetamine grew to be so large that in 1970, Congress passed the Controlled Substances Act. This law introduced a scheduling system

that placed various drugs of abuse into categories I-V, based on the drug's medical value, harmfulness to the user and the environment, and its potential for dependence and/or misuse and abuse.[7,17,20] Schedule I drugs, like heroin and marijua, have no accepted medical value but a high potential for abuse.[20] Schedule II drugs, such as meth and amphetamine, have a high potential for abuse and dependence but some therapeutic uses.[7,17,20] Prescription drugs are classified as Schedule III-V drugs; these drugs can be obtained through prescription refills.[20]

2.3. Illegal Production of Methamphetamine and Amphetamine

As the United States government placed new rules and regulations on methamphetamine and amphetamine use, illegal laboratories and production methods emerged. One illegal production method uses phenylacetone as the starting material.[7,16-17] This method is called the P2P method, in which phenylacetone is combined with methylamine and reduced to result in a 50/50 mixture of d- and l-meth.

The United States government eventually classified phenylacetone as a Schedule II drug due to its use in the illegal production of meth.[17] As a result, meth cooks had to find new starting materials to use to produce meth. Ephedrine and pseudoephedrine, common ingredients found in prescription cold medicines, became those new starting materials. With these new starting materials, new illegal production methods emerged: the Red Phosphorous and Birch reduction methods. The Red Phosphorous method involves the reduction of l-ephedrine or d-pseudoephedrine with hydroiodic acid and red phosphorous acid to reduce the starting molecules to result in pure d-meth. [7,16-17] Red phosphorous can be obtained from common materials such as matchbook striker plates, while hydroiodic acid can be created from iodine. [7,16] The Birch reduction method uses the same starting materials as the Red Phosphorous method, with lithium metal in liquid ammonia to result in pure d-meth.[7] Lithium metal can be obtained from lithium batteries, and ammonia from agricultural sources.[7] Phenylpropanolamine can be substituted as the

starting material for either the Red Phosphorous or Birch reduction methods to result in d-amphetamine instead of meth.[16]

In 2006, Congress passed the Combat Methamphetamine Epidemic Act, which aimed at reducing the availability of pseudoephedrine and ephedrine as starting materials for illegal meth production.[14,21] As part of the act, pharmacies are required to record the number of store bought medicine packages that contained either ephedrine, pseudoephedrine, or phenylpropanolamine, for up to two years.[17] Later, many state pharmacies removed these packages from the shelves and placed them behind the counter. To this day, many states require a driver's license and a signature for the purchase of these products.

Despite the growing limitations placed on the availability of pseudoephedrine and ephedrine, meth cooks adapted to the situation and began to produce meth using the One-Pot method. This method, a modified version of the Birch reduction method, uses crushed pseudoephedrine tablets as the starting material with lithium metal and ammonia to result in d-meth.[22] The materials needed for the reaction process can be easily obtained from any local store and placed into small, carriable containers. Meth cooks then only need to continually shake and release the built-up gases inside the container to yield d-meth.

2.4. Enantiomers

Methamphetamine and amphetamine enantiomers are chiral compounds, in other words, compounds that are non-superimposable mirror images, as seen in Figures 1-2.[4] These chiral chemical compounds have two optical isomers, called enantiomers: d(+)-dextrorotatory and l(-)-levorotatory.[4-5,11,16,23] Enantiomers are compounds that have the same physical and chemical properties, but the only difference between them is the direction the drug or chemical rotates in polarized plane light, as seen in Figures 1-2.[4] Dextrorotatory compounds rotate polarized light to the right and are "right-handed."[7] Levorotatory compounds, on the other hand, rotate polarized

light to the left and are "left-handed."[7] Enantiomers are chemical compounds that are mirror images of each other and can exist independently but also equally as racemates.

2.4.1. Pharmacology

When meth and amphetamine are used, these drugs can cause increased alertness, euphoria, and a general sense of well-being in the user by increasing the norepinephrine and dopamine levels in the central nervous system (CNS).[7,16,24] When either drug is smoked or injected, the effects will be almost instantaneous.[7] Meth and amphetamine use can also produce the "fight-or-flight" response associated with the peripheral nervous system.[7,16] The fight-or-flight response is characterized by an increased heart rate, blood pressure, and glucose blood levels.[16] When either drug is taken in high doses, serious health complications can develop like irregular heartbeat, skin tremors, or even heart attacks.[16] The enantiomers of either drug will affect the body in separate ways depending on the form. The d-isomers are more physiologically active in the CNS, which means that are more likely to be abused.[7,23] The d-form of meth is referred as street meth because it is five times more reactive than the l-form.[4] This form of meth is the active ingredient in decongestant medicines like Vicks® VapoInhaler™ because it is more physiologically active in the peripheral nervous system.

2.5. Analytical Methods

The separation and quantification of the chiral enantiomers of meth and amphetamine is not a new topic in forensic science. As technology advances and becomes more efficient, more specific analytical techniques for the separation and quantification of drug enantiomers will emerge. Most drug screening methods that are employed in forensic and clinical toxicology labs today are geared towards identifying the more physiologically active d-enantiomer for either drug.[23]

These screening methods will not identify the difference between either the d- or l-meth and/or amphetamine enantiomers, but only if an analyzed sample screened positive for the

presence of meth or amphetamine.[7,11] These positively-screened results need to be confirmed by a second analytical method. Two confirmatory techniques that are commonly used for the chiral separation of enantiomers, gas chromatography and liquid chromatography, will be discussed. A variety of detectors can be used with either technique, but this paper will focus on mass spectrometry (MS) as the detector of choice. There are three common methods that are employed for the enantiomeric separation of chiral enantiomers; only the two most advantageous methods for our clinical lab will be discussed.

2.5.1. Gas chromatography

Gas chromatography, or GC, combined with a mass spectrometer (MS) detector, has been the instrument of choice for the separation of meth and amphetamine enantiomers.[4,7,11] However, there is a distinct disadvantage to using this technique. During analysis, drug samples are exposed to extreme heat to volatilize them for analysis. Many drugs and substances become unstable at elevated temperatures. GC-MS is typically performed using a chiral derivatization reagent, which is added to a sample prior to column analysis, to create diastereomers.[6] These reagents are special chemicals that are added to a sample to make them more stable for analysis. However, chiral reagents can affect the percentages of meth and amphetamine enantiomers in samples of suspected abusers. This technique can also produce relatively high error rates and cause racemization of enantiomers to occur.[6]

2.5.2. Liquid chromatography

Liquid chromatography, or LC, combined with two MS detectors (LC-MS/MS), is becoming the staple technique in forensic and clinical toxicology labs due to its ability to detect very low levels of drugs and substances in samples. LC methods often require less sample preparation and can be used with or without a chiral derivatization reagent. When compared to

GC-MS, LC-MS/MS can lead to more accurate results because racemization of enantiomers is not as likely to occur since pre-column derivatization is not used.[6]

With LC-MS/MS, there are three common methods that are used for the enantiomeric separation of meth and amphetamine. The first method involves the use of a chiral derivatization reagent to form diastereomers for the analysis of enantiomers on an achiral column.[4,25] The second method a forensic and clinical toxicology lab can consider is with a chiral column. [4,25] The third method for the enantiomeric separation of meth and amphetamine enantiomers is the addition of a chiral selector as an additive in a mobile phase. [4,25]

A chiral column will eliminate the need for derivatization of a sample prior to injection. The sample will undergo preparation and then it will be injected directly onto the column for analysis. The enantiomers, when introduced to the column, will react differently with the internal environment to allow for separation of enantiomers to be achieved.[5] There are many published LC methods using chiral columns to separate meth and amphetamine enantiomers.[5-6,11-14] Several types of commercial chiral column stationary phases are available, including cyclodextrin, chiral ligand exchange, and macrocyclic glycopeptides.[26-27] Vancomycin is an example of a macrocyclic glycopeptide chiral stationary phase, often seen in Astec Chirobiotic™ columns. Vancomycin is a chiral stationary phase that consists of a non-sugar portion of various fused macrocyclic rings surrounded by connected portions of carbohydrate molecule groups, bounded to fused silica.[27] The Astec Chirobiotic™ column from Sigma-Aldrich has effectively separated and quantified both meth and amphetamine enantiomers in common biological specimens of forensic interest, including urine, blood, and oral fluid.[5,7,12-13,15]

A major disadvantage of using chiral columns is that they are often very expensive. Forensic and clinical toxicology labs, which have strict budgets or not much in the way of

available funding, may find themselves unable to adopt of a method with such an expensive component.

The expense of chiral columns means that many forensic and clinical toxicology labs look for more cost-effective separation methods. One such method is the use of derivatization with a chiral derivatization reagent, which is much less expensive. Studies show that reagents like 1-fluoro-2,4-dinitrophenyl-5-L-aniline amide (Marfey's reagent) and 9-fluorenylmethly chloroformate-L-proline (FMOC-L-Pro) are effective chiral derivatization reagents for the separation of methamphetamine and amphetamine enantiomers.[4,7,14-15] The addition of the chiral derivatization reagent to an enantiomer will form diastereomers that can be analyzed using standard achiral chromatography, including normal and reversed phase means. For normal phase HPLC, the stationary phase is made up of polar packing materials, like silica. By increasing the strength of the nonpolar mobile phase, the least polar analytes will elute off the column first, followed by the more polar analytes. Reversed phase HPLC works in the exact opposite manner; the stationary phase is made up of nonpolar packing materials, like C_{18} or C_8 chains. By increasing the strength of the polar mobile phase, the least nonpolar analytes elute off the column first, followed by the more nonpolar analytes.

2.6. Extraction Methods

Two extraction methods that are commonly employed in forensic and clinical toxicology labs for chiral separation of d- and l-enantiomers of meth and amphetamine include liquid-liquid extraction and solid phase extraction.[6-7,11-12,14-15,28-34]

2.6.1. Liquid-Liquid Extraction Process

Liquid-liquid extraction, or LLE, is an analytical extraction process where an immiscible extraction solvent is added to a sample and the aqueous phase (usually water) and the organic phase are mixed together by shaking to allow for the separation of analytes to occur between the

two phases. The phase that contains the analytes of interest is removed, the extraction solvent is then dried down, resuspended in a polar organic solvent, and injected onto the instrument.

2.6.2. Solid Phase Extraction Process

Solid phase extraction, or SPE, is an alternative extraction process to the LLE process described above. By using a cartridge column device that is filled with solid particle packing material (stationary phases), liquid samples can be chemically divided into analyzable parts through various elution steps.[35-36] Figure 3 shows an example of the overall SPE process described.[36]

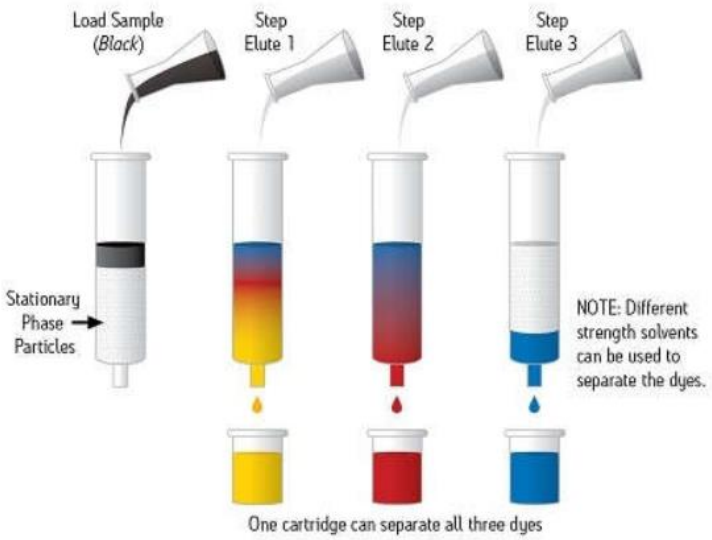

Figure 3: SPE process[36]

2.7. Conclusion

Methamphetamine and amphetamine are CNS stimulant drugs that have a long history of abuse. With increasing rules and regulations placed on the common starting materials used in the illegal production of meth and amphetamine, there have been a variety of methods developed. These methods include P2P, Red Phosphorous, Birch reduction, and the One-Pot methods. The

P2P method will result in a racemic mixture of both d- and l-enantiomers of meth. The Red Phosphorous and Birch reduction methods can sometimes result in d-amphetamine, depending on the starting material used. The Red Phosphorous, Birch reduction, and One-Pot methods will result in d-enantiomer of meth.

The separation and quantification of meth and amphetamine enantiomers is not a new problem in forensic and clinical toxicology labs. Many labs will employ screening methods that cannot tell the difference between either enantiomer; only if an analyzed sample screens positive for the presence of meth and/or amphetamine. These positive samples should be sent to an off-site lab for the separation analysis. The process of sending the positive samples to another lab for the separation analysis can get expensive in the long run. While GC and LC are common techniques used for the analysis of drug enantiomers, LC is becoming more popular. Many LC studies use an expensive chiral column that can eat a lab's bottom-line profits.

The purpose of this research was to develop a simple and cost-effective chiral separation method to separate d- and l-meth and amphetamine in urine samples using LC-MS/MS. Three separation methods were developed: two using an Astec Chirobiotic chiral column, and one using an achiral column to determine which chiral method was more effective at enantiomeric separation for either drug. Through this research, the Oklahoma State University Clinical Laboratory Services will have the ability to conduct the separation analyses of meth and amphetamine enantiomers in-house.

CHAPTER III

III. METHODOLOGY

3.1. Overview

The purpose of this project was to develop and compare a cost-effective a chiral separation method that separated the d- and l-enantiomers of methamphetamine (meth), and its primary metabolite, amphetamine (amp), via LC-MS/MS. This chapter outlines three separation methods were developed: two using a chiral column and one using Marfey's reagent for pre-column derivatization. The separation power of each method was evaluated to determine which method was more effective at the separation of the d- and l-enantiomers of both meth and amp in urine.

Topics discussed within this chapter include: chemicals and equipment used for each separation method, instrumentation, and the LC-MS/MS separation parameters determined for each method. The sample extraction processes for the chiral column and the pre-column derivatization methods, along with the derivatization process with Marfey's reagent, are also discussed. Other topics include the data analysis for each method and future validation.

3.2. Chemicals and Equipment

3.2.1. Materials

d-amphetamine, l-amphetamine, ±-amphetamine, d-methamphetamine, l-methamphetamine, ±-methamphetamine (1 mg/mL), and internal standards ± d_5-methamphetamine and ± d_6-amphetamine (1 mg/mL) were purchased from Cerilliant (Round Rock, TX). ACS-grade ammonium hydroxide was purchased from BDH (West Chester, PA). ACS-grade hydrochloric acid was purchased from Fisher Scientific (Waltham, MA). ACS-grade methanol was purchased from J.T. Baker (Center Valley, PA). ACS-grade ethyl acetate was purchased from Honeywell (Muskegon, MI). Ammonium formate was purchased from Alfa Aesar (Ward Hill, MA). ACS-grade sodium bicarbonate was purchased from J.T. Baker (Phillipsburg, NJ). Marfey's reagent was purchased from TCI (Portland, OR). ACS-grade acetone, glacial acetic acid, and formic acid were purchased from EMD (Billerica, MA). Deionized pure water was produced on-site with a Thermo Scientific Barnstead Nanopure water purification system from ThermoFisher Scientific (Asheville, NC).

3.2.2. Reagents and Solutions

A variety of solutions were needed throughout each separation method. For the chiral column solid phase extraction (SPE) and dilute and shoot (D & S) extraction methods, a solution of 0.1 % glacial acetic acid + 0.02 % ammonium hydroxide in methanol was prepared for the mobile phase solution. A hydrolysis solution was prepared for the D & S extraction method, containing internal standards, 0.1 M pH 4.0 acetate buffer, and water. A sample diluent solution of 95:5 water/methanol was prepared weekly.

For the pre-column derivatization reagent SPE separation method, a gradient elution was used with a solution of 2mM ammonium formate + 0.1 % formic acid mobile phase solution prepared in both methanol and water. Additional solutions included 0.1 % Marfey's reagent in

acetone and 1.0 M methanolic hydrochloric acid in water. The Marfey's reagent was stored at 4°C in an amber bottle for up to one month.[7]

For both the SPE separation methods for the chiral column and the pre-column derivatization method, an elution solution of 98:2 ethyl acetate/ammonium hydroxide was prepared fresh daily and stored at room temperature. A 1.0 M sodium bicarbonate in water solution was prepared. A 500 ng/mL working solution of each analyte, one with both meth enantiomers, and one with both amphetamine enantiomers, listed in the materials section, was made up separately in urine.

3.2.3. Instrumentation

All samples were analyzed with a Shimadzu HPLC system from Shimadzu Corporation (Kyoto, Japan). The HPLC system consists of a system controller, CBM-20A, a solvent delivery unit, LC-20AD, an auto-sampler, SIL-20AC, and a column oven, CTO-20AC.

The Shimadzu HPLC system is attached to an Applied Biosystems 4000 Q-Trap LC-MS.MS System from AB Sciex (Foster City, CA). The mass spectrometer is equipped with a Turbo V^{TM} electrospray ionization source, a Harvard Apparatus syringe pump (Holliston, MA) and a Genius^{2} 3020 Nitrogen generator from Peak Scientific (Billerica, MA). Analyst 1.6.2 Software was used to control the instrument, sample data collection, and analysis.

3.3. Urine Samples

Five anonymized urine samples were provided by OSU-CLS in Tulsa, Oklahoma for testing. Four samples were negatively confirmed for meth, while one sample was positive for meth.

3.4. Chiral Separation Method Development

3.4.1. Chiral Column Method

3.4.1.1. Sample Extraction Methods

3.4.1.1.1 Dilute and Shoot

The dilute and shoot (D & S) method is an extraction method, where a sample is diluted prior to injection onto an instrument, that is often employed in forensic and clinical toxicology labs. Prior to any sample workup, an internal standard solution of 1 µg/mL internal standard, 0.1 M pH 4.0 acetate buffer, and water was made, and then centrifuged for 10 minutes at 13,000 rpm. To a 2 mL Eppendorf tube, 50 µL of the 500 ng/mL working urine solution sample + 50 µL internal standard solution were added. The samples were vortexed for 10 seconds before the addition of 150 µL of a 95:5 water/methanol. The samples were vortexed for 10 seconds, then centrifuged for 10 minutes at 13,000 rpm. Next, 200 µL of the supernatant was transferred to an injection vial prior to instrumental analysis.

3.4.1.1.2 Solid Phase Extraction

The solid-phase extraction (SPE) method was adapted from SPEware Corporation.[37] Trace-N SPE columns from SPEware were used. To wash the SPE columns to prepare them for the addition of the sample solution, 2 mL ethyl acetate was pulled through the columns under a vacuum into waste at a flow of 1.0 mL/min. The process was repeated with 2 mL methanol, then with 1 mL water. Next, 50 µL of the 500 ng/mL working urine solution was added to its labeled column and pulled through with a vacuum at a flow rate of 1.0 mL/min.

The columns were washed again with 2 mL 1.0 M sodium bicarbonate, then with 2 mL water. Next, the columns were dried for 6 minutes at 80 psi. Next, the d/l-meth and amp enantiomers were eluted from the columns with 2 mL 98:2 ethyl acetate/ammonium hydroxide

elution solution. The elution tubes were evaporated to dryness for 30 minutes at 45 °C under a stream of nitrogen. Next, the samples were resuspended in 200 µL 0.1 % glacial acetic acid + 0.02 % ammonium hydroxide in methanol. The samples were vortexed for 10 seconds, and then 180 µL of the solution was transferred to an injection for instrumental analysis.

3.4.1.2. LC-MS/MS Parameters

The LC-MS/MS conditions were optimized to achieve baseline separation of d/l-meth and d/l-amp using an Astec CHIROBIOTIC V2 Chiral column (25 cm x 2.1 mm, 5 µm) under isocratic conditions with 100 % 0.1 % glacial acetic acid + 0.02 % ammonium hydroxide in methanol for 13 minutes. The oven temperature was set to 25 °C with a flow rate of 0.25 mL/min and an injection volume of 2 µL. Mass spectrometry data was acquired using ESI positive mode. The gas temperature was set to 350 °C and the capillary voltage set to 1000 V. Multiple reaction monitoring mode (MRM) was chosen to allow multiple user defined ion fragments for each enantiomer to be monitored. MRM transitions and MS/MS details can be seen in Table 1. The retention times for each extraction method for each analyte are listed in Table 2.

Table 1: MRM values & MS/MS details for the chiral column method. The precursor and product ions m/z (Q1 & Q3), declustering potential (DP), collision energy (CE), and collision exit potential (CXP) values for d- and l-meth and amp enantiomers are listed.

Analyte	Q1 (Da)	Q3 (Da)	DP (Volts)	CE (Volts)	CXP (Volts)
Methamphetamine	150.10	91.00	56	25	14
Methamphetamine	150.10	119.00	56	15	4
Methamphetamine-D5	155.00	91.10	60	20	4
Amphetamine	136.20	91.00	36	25	14
Amphetamine	136.20	119.00	36	13	18
Amphetamine-D6	142.10	125.10	41	13	6
l-Amphetamine	136.10	91.00	21	23	14
l-Amphetamine	136.10	119.00	36	10	18
d-Amphetamine	136.15	91.11	1	21	14
d-Amphetamine	136.15	119.00	36	10	18
l-Methamphetamine	149.667	91.20	1	19	10
l-Methamphetamine	149.667	119.10	1	15	18
d-Methamphetamine	150.094	90.90	101	29	14
d-Methamphetamine	150.094	118.90	101	15	18

Table 2: Retention times and MS/MS details for the chiral column method extraction methods. The precursor and product ions m/z (Q1 & Q3) and retention times (RT) for d- and l-meth and amp enantiomers are listed. Since two peaks were observed for all analytes, the averaged retention times between each observed peak are listed.

Analyte	Q1 (Da)	Q3 (Da)	SPE RT (min)	D & S RT (min)
Methamphetamine	150.10	91.00, 119	9.25	10.23
Methamphetamine-D5	155.00	91.10	-	8.61
Amphetamine	136.20	91.00, 119	7.97	7.65
Amphetamine-D6	142.10	125.10	-	8.61
l-Amphetamine	136.10	91, 119	7.95	7.64
d-Amphetamine	136.15	91.11, 119	9.94	7.63
l-Methamphetamine	149.667	91.20, 119.10	9.70	8.57
d-Methamphetamine	150.094	90.90, 118.90	9.83	8.46

3.4.2. Pre-Column Derivatization Reagent Method

3.4.2.1. Sample Extraction

The solid-phase extraction (SPE) method was adapted from SPEware Corporation, as described in section 3.4.1.1.2.[37] The derivatization method was adapted from Mitchell[7] and Newmeyer et al.[15] After evaporation under a stream of nitrogen for 30 minutes at 45°C, the samples were resuspended in 100 µL water + 20 µL of 1.0 M sodium bicarbonate, and then vortexed for 10 seconds. Next, 100 µL of 0.1 % Marfey's reagent in acetone was added, and then the tubes were vortexed for 10 seconds to mix the contents together.

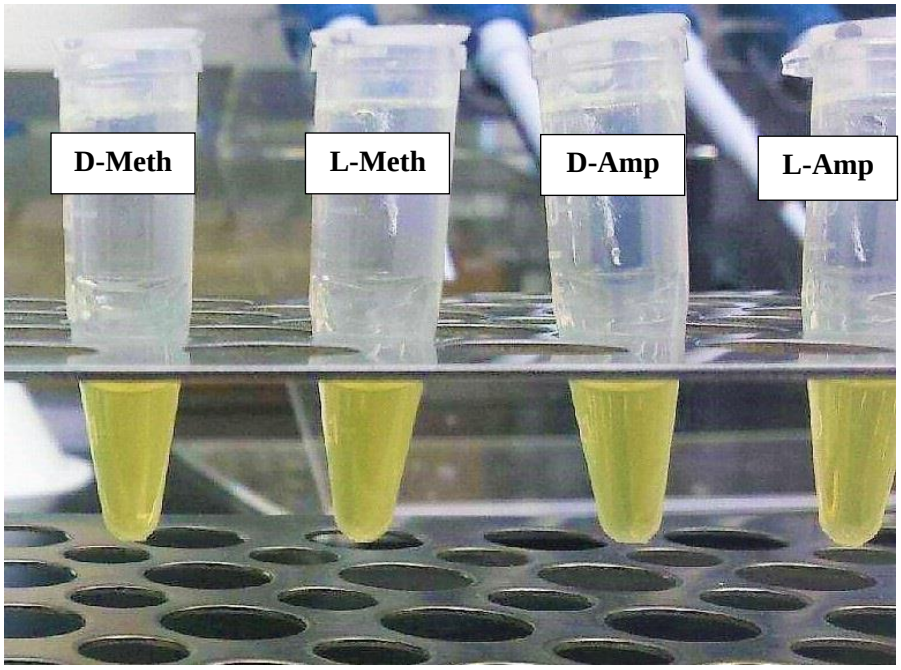

Figure 4: Marfey's reagent in acetone is bright yellow

Then, the samples were heated at 45°C for 1 hour. After 60 minutes, the samples are cooled to room temperature before the addition of 40 µL 1.0 M hydrochloric acid, and then vortexed for 5 seconds.

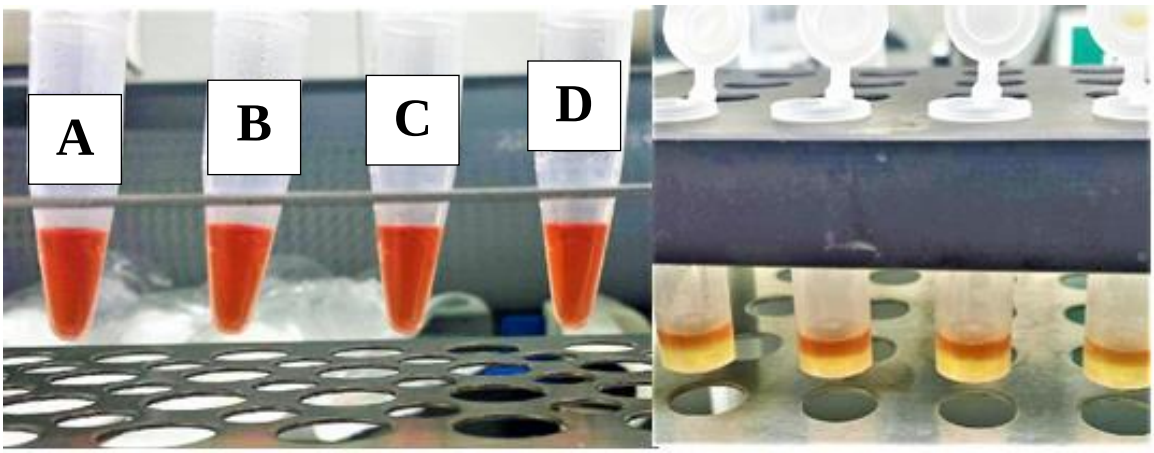

Figure 5. (Left) The heating process will cause the samples to change from yellow to red. The samples are labeled as: d-meth (A), l-meth (B), d-amp (C), and l-amp (D). (Right) The addition of HCl will create the yellow bottom layer

Then, the samples are taken to dryness under a stream of nitrogen for 2 hours at 45°C. Next, the samples are resuspended in 200 µL of 2 mM ammonium formate + 0.1 % formic acid in methanol. Next, the samples were vortexed for 10 seconds before centrifuging for 5 minutes at 2800 rpm. Then, 180 µL of the supernatant was transferred to an LC injection vial for instrumental analysis.

3.4.2.2. LC-MS/MS Parameters

The LC-MS/MS conditions were optimized in the hopes of achieving baseline separation of d/l-meth and d/l-amp using an Restek Raptor Biphenyl achiral column (50 x 2.1 mm, 2.7 µm) with a guard column under binary flow conditions with 7.2 % mobile phase B (2 mM ammonium formate + 0.1 % formic acid in methanol) and 92.8 % mobile phase A (2 mM ammonium formate + 0.1 % formic acid in water) for 8 minutes. A time program was used to vary the pump B % concentration. For 3.50 minutes, the pump B %concentration was held at 7.2 %, then increased to 35 % at 3.51 minutes. The pump B % concentration was then held at 35 % until 4.51 minutes, where it decreased back to 7.2 %. The total run time was 12 minutes. The oven temperature was

set to 30 °C with a total flow rate of 0.70 mL/min with an injection volume of 2 µL. Mass spectrometry data was acquired using ESI positive mode. MRM transitions and MS/MS details can be seen in Table 2. Only retention times for the un-derivatized meth and amp analytes are noted below, because the derivatives of each enantiomer were not observed at this time.

Table 3: MRM values & MS/MS details for the pre-column derivatization method. The same descriptions listed for Table 1 are also used.

Analyte	Q1 (Da)	Q3 (Da)	DP (Volts)	CE (Volts)	CXP (Volts)	RT (min)
Derivatized d-Amphetamine	386.10	136.10	46	19	6	-
Derivatized d-Amphetamine	386.10	91.10	46	19	6	-
Derivatized d-Methamphetamine	400.20	150.10	56	17	12	-
Derivatized d-Methamphetamine	400.20	91.10	56	69	24	-
Derivatized l-Amphetamine	386.10	136.10	46	19	6	-
Derivatized l-Amphetamine	386.10	91.10	46	19	6	-
Derivatized l-Methamphetamine	400.20	150.10	56	17	12	-
Derivatized l-Methamphetamine	400.20	91.10	56	69	24	-
Methamphetamine	150.10	91.00	56	25	14	4.23
Methamphetamine	150.10	119.00	56	15	4	4.23
Amphetamine	136.20	119.00	36	13	18	2.71
Amphetamine	136.20	91.00	36	25	14	2.70
Methamphetamine-D5	155.00	91.10	60	20	4	5.15
Amphetamine-D6	142.10	125.10	41	13	6	4.59

3.5. Method Validation

Since for this project, were only interested in the detecting the presence of d or l meth and/or amp, a quantitative multi-point calibration curve was not used at this time. Method validation studies according to SWGTOX guidelines will be conducted by the OSU-CLS prior to clinical incorporation of this method.[38] Only the efficiency of the chiral column separation methods was investigated.

3.6. Data Analysis

Data were analyzed using the Analyst 1.6.2 software to determine the retention times of the peaks and their widths for the D & S and SPE separation methods for the chiral column. Using Microsoft Excel, the average resolution or R-values, for each day of experiments was determined for the meth and amp mixture samples. The average R-values were calculated using the Analyst software generated values and were compared to the values determined by hand. GraphPad Prism software (San Diego, CA) was used to calculate the p-values and the 95% confidence intervals between the chiral column extraction methods for each drug to determine which extraction technique was more efficient at the enantiomeric separation.[38]

CHAPTER IV

IV. RESULTS

4.1. Presentation of Results

4.1.1. Chiral Column Results

Baseline separation was achieved for both the SPE and D & S extraction methods developed for the chiral column for amphetamine only. Baseline separation was achieved for methamphetamine for the SPE method, but not for the D & S. Figure 6 shows the baseline separation of d- and l-enantiomers of both meth and amphetamine in a spiked 500 ng/mL urine sample extracted with the SPE method for one sample run. The d-enantiomer always eluted off the column first, followed soon after by the l-enantiomer for both methamphetamine and amphetamine for both extraction methods. The average retention times for the methamphetamine and amphetamine enantiomers are listed in Table 2 for the chiral column extraction methods.

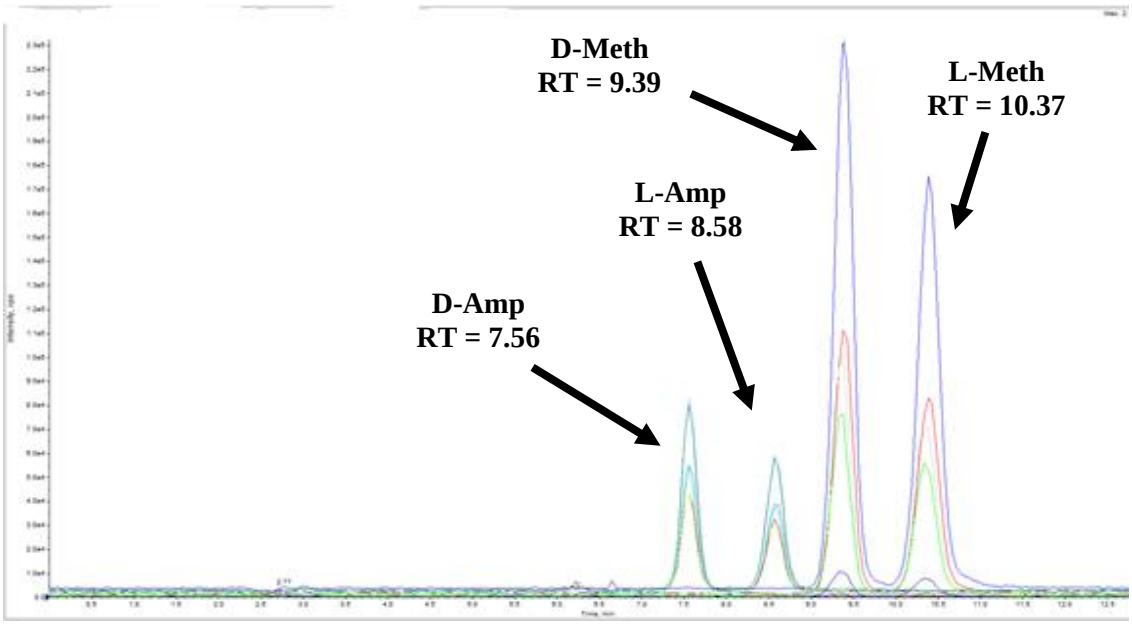

Figure 6: Baseline separation of d- and l-amphetamine and methamphetamine enantiomers with SPE extraction method

Figure 7 is an example of the spiked 500 ng/mL urine sample extracted with the D & S extraction method showing baseline separation of d- and l-enantiomers of amphetamine. Figure 8 is the extracted urine sample described for Figure 7, showing separation of d- and l-enantiomers of methamphetamine.

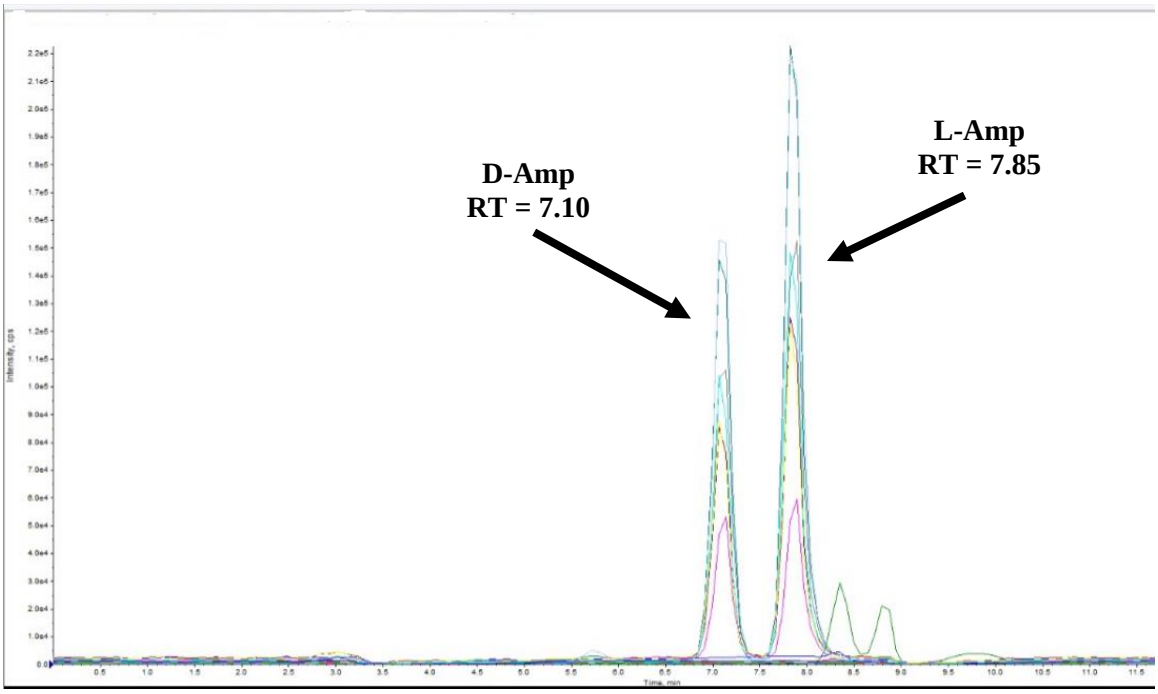

Figure 7: Separation of d- and l-amphetamine enantiomers with D & S extraction method

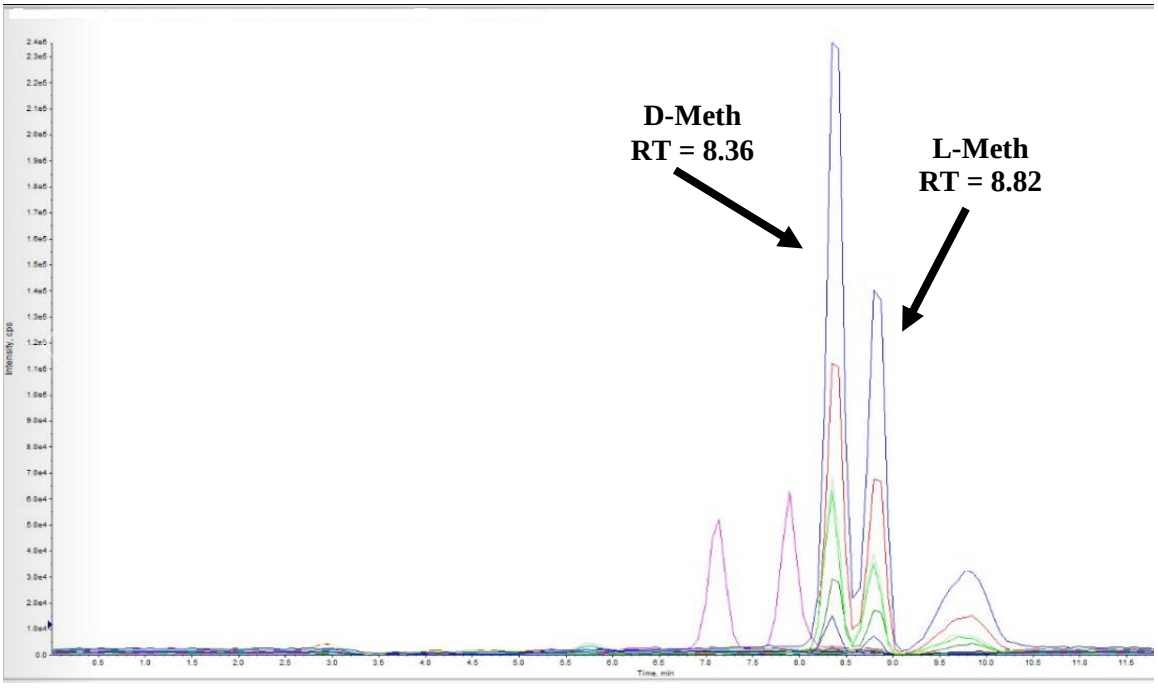

Figure 8: Baseline separation of d- and l-methamphetamine with D & S extraction method

The Analyst 1.6.2 software was used to determine the retention times of the observed peaks and their widths for both extraction methods. The average R-values were calculated using the resolution equation,[39]

$$R = \frac{(t_{rb} - t_{ra})}{(\frac{1}{2})(W_{ba} + W_{bb})}$$

Where R is the resolution, t_r is the peak retention time; t_{ra} for peak a, t_{rb} for peak b, and w_b is the average width of the peak at the base; w_{ba} for peak a, w_{bb} for peak b. The average R-values were calculated using the Analyst calculated values and were compared to the values determined by hand. This data is presented in Tables 4 and 5. Table 4 are the SPE extraction R-values using the Analyst computer generated values (CPU) and the hand-generated values (Hand). Table 5 are the D & S extraction R-values using the Analyst generated values and the hand-generated values. A single sample was extracted once for each extraction method and run over the course of 3 days. The average R-values for each drug for an extraction method were calculated and reported.

Table 4: Chiral Column - SPE Extraction R-values.

Days	CPU Meth Mix	CPU Amp Mix	Hand Meth Mix	Hand Amp Mix
Day 1	0.87	1.15	0.94	1.21
Day 2	0.90	0.94	0.92	0.99
Day 3	0.87	0.84	0.93	0.83
Average	0.88	0.98	0.93	1.01

Table 5: Chiral Column - D & S Extraction R-values.

Days	CPU Meth Mix	CPU Amp Mix	Hand Meth Mix	Hand Amp Mix
Day 1	0.78	0.72	0.88	0.90
Day 2	0.73	0.75	0.77	0.75
Day 3	0.67	0.80	0.70	0.96
Average	0.72	0.76	0.79	0.83

A total of 5 unknown extracted urine samples provided from OSU-CLS, labeled A-E, were analyzed over the period of 2 days. The samples fell into two broad categories: positive for methamphetamine or negative for methamphetamine. For all the analyzed samples, the presence of amphetamine was determined. One sample was positive for methamphetamine, specifically d-methamphetamine and d-amphetamine, while the other four samples were negative for meth and amphetamine. These unknown samples were compared to the retention time of the 1 µg/mL Cerilliant standards listed in Table 6.

Table 6: Cerilliant standards (1 µg/mL in methanol) retention times

Cerilliant Standards	Retention Times (min)
d-Amphetamine	7.31
l-Amphetamine	8.27
d-Methamphetamine	9.04
l-Methamphetamine	9.97
±-Amphetamine	7.57, 8.58
±-Methamphetamine	9.38, 10.37

The retention times for each standard was obtained with the optimized LC-MS/MS chiral column parameters listed in the methodology chapter. Figure 9 shows the chromatogram for Unknown E that was negative for meth and amphetamine.

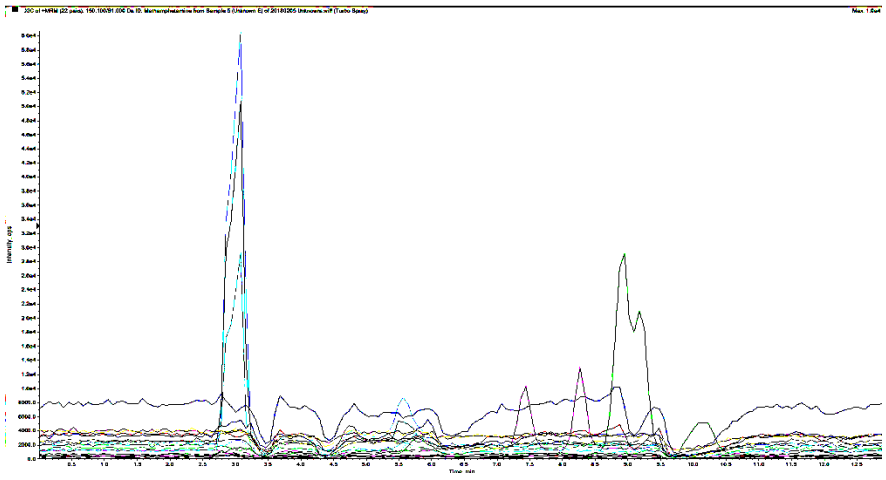

Figure 9: Unknown sample E negative for amphetamine and methamphetamine

Figure 10 shows the chromatogram for Unknown D that was positive for methamphetamine. When the observed peak was compared to the retention times standards in Table 8, it was determined that this unknown sample was positive for d-methamphetamine, at 8.91 minutes. There is a small peak observed around 7.33 minutes that was determined to be d-amphetamine.

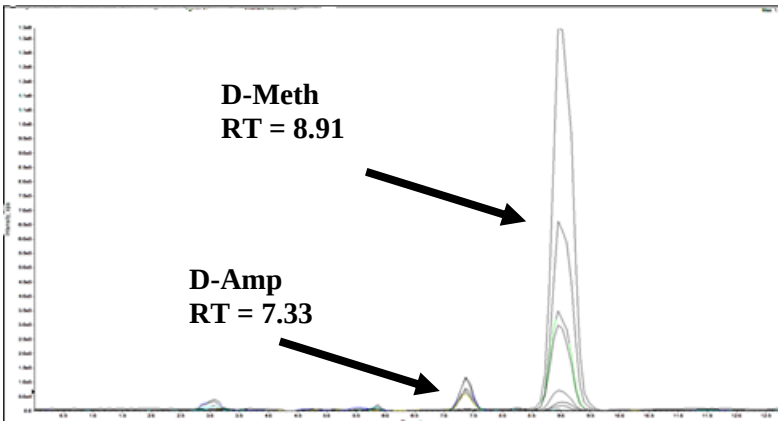

Figure 10: Unknown sample D that was positive for d-meth and d-amp

4.1.2. Pre-Column Derivatization Results

There was no significant difference between the 500 ng/mL derivatized and the un-derivatized urine samples, when using the conditions listed in the methodology chapter, using a mobile phase of 2 mM ammonium formate + 0.1 % formic acid in methanol (mobile phase B) or water (mobile phase A). Figure 11 is the un-derivatized 500 ng/mL urine sample, that shows the ion ratios of d/l-amphetamine and d/l-methamphetamine, labeled A-D. Figure 12 is of the derivatized 500 ng/mL urine sample, with the same letter labels as Figure 11.

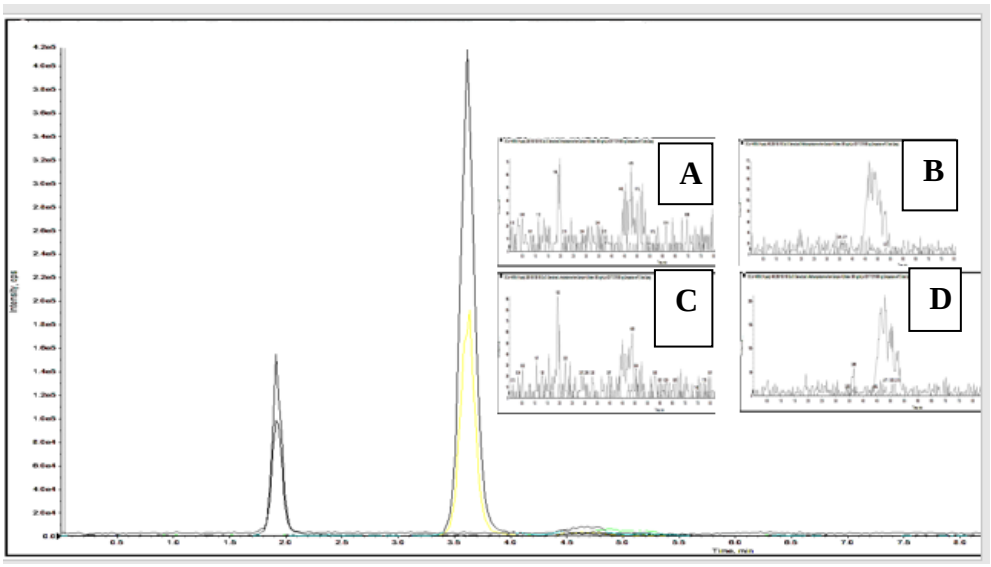

Figure 11: Underivatized 500 ng/mL urine sample, showing relative ion ratios**.** A: Derivatized d-amp. B: Derivatized l-amp. C: Derivatized d-meth. D: Derivatized l-meth

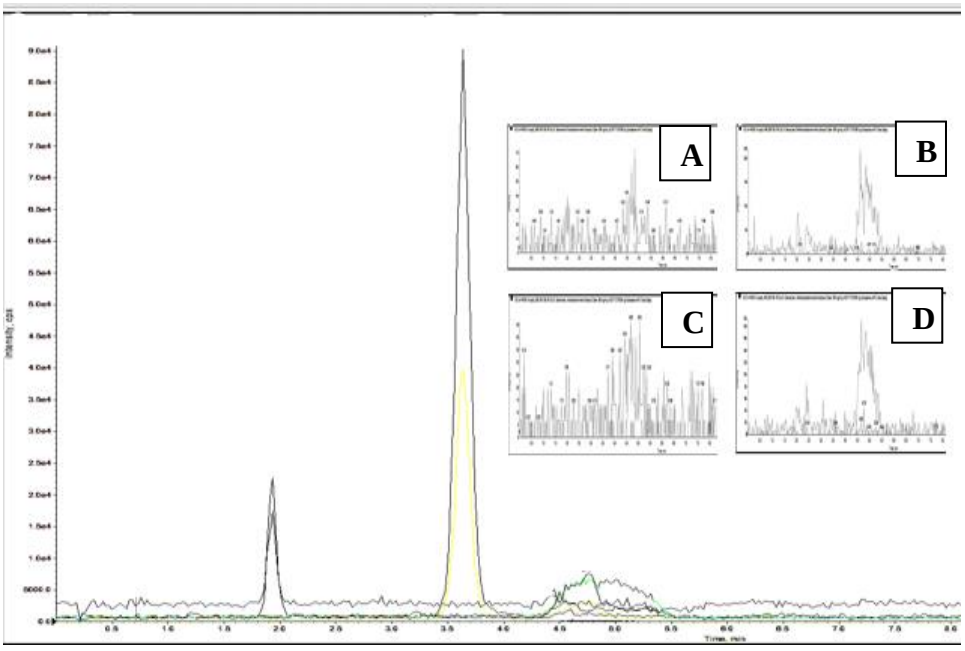

Figure 12: Derivatized 500 ng/mL urine sample showing relative ion ratios. A: Derivatized d-amp. B: Derivatized l-amp. C: Derivatized d-meth. D: Derivatized l-meth

CHAPTER V

V. DISCUSSION

5.1 Discussion

5.1.1. Chiral Column Separation Method Discussion

The purpose of this research was to develop a cost-effective chiral separation method using an Astec Chirobiotic chiral column, to separate d- and l-meth and amphetamine in urine samples using LC-MS/MS. A separation method using Marfey's chiral derivatization reagent was also attempted for an achiral column. The research hypothesized that the SPE extraction method was going to be more efficient at the enantiomer separation for both drugs when compared to the D & S method for the chiral column. Baseline separation for both chiral column extraction methods would also be achieved. The research also hypothesized that baseline separation of the derivatized enantiomers for both meth and amphetamine would be achieved with pre-column derivatization with Marfey's chiral derivatization reagent.

The overall results were mixed, in regards with the stated hypotheses. The p-value for the ANOVA comparison test of the average R-values from tables 4 and 5 for meth is $p = 0.0067$, $F = 8.693$. Therefore, when $\alpha = 0.05$, $p < \alpha$, there is sufficient evidence to partially disprove the hypothesis that the SPE extraction method would be more efficient at the enantiomeric separation of meth when compared to the D & S extraction method.

There is a statistical difference observed between the average R-values calculated using either resolution calculation method between the two chiral column extraction methods for meth.

The p-value for the ANOVA comparison test for amphetamine is $p = 0.1377$, $F = 2.456$. When $\alpha = 0.05$, $p > \alpha$, there is insufficient evidence to reject the hypothesis that the SPE extraction method would be more efficient at the separation of amp enantiomers compared to the D & S extraction method. There is no statistical difference observed between either resolution calculation for amp when using either extraction method.

A Tukey's multiple comparisons test was conducted to investigate where the differences are between the experimental conditions of the average R-values between the SPE and D & S extraction methods obtained using either the Analyst computer generated values or by hand. This was done to determine if there is a significant difference between using the Analyst software generated R-values and the hand-calculated R-values for either drug between the two extraction methods used for the chiral column. Table 7 shows the 95% confidence interval values and the multiple comparisons means that were generated using GraphPad Prism software for meth. Table 8 lists the p-values for the pairwise comparisons when $\alpha = 0.05$ for meth. A result is significant if $p \leq \alpha$.

Table 7: Meth Multiple comparisons 95% confidence intervals (CI). CPU are the Analyst software R-values, while Hand are the R-values using the SPE and D & S average R-values from tables 4 and 5.

Method	Lower 95% CI	Upper 95% CI
CPU SPE[B]	0.84	0.92
Hand SPE[A,C]	0.91	0.95
CPU D & S[B,C]	0.59	0.86
Hand D & S[A]	0.56	1.01

Table 8: Meth Pairwise comparisons p-value & 95% confidence intervals. CPU are the Analyst software R-values, while Hand are the R-values using the SPE and D & S average R-values from tables 4 and 5. A result is significant if $p \leq \alpha$, when $\alpha = 0.05$.

Method Pair	Adjusted p-value	95% CI	Significant?
CPU D & S vs Hand D & S	0.5960	-0.198 to 0.0845	No
CPU D & S vs CPU SPE[B]	0.0340	-0.295 to -0.0121	Yes
CPU D & S vs Hand SPE[C]	0.0075	-0.345 to -0.0621	Yes
Hand D & S vs CPU SPE	0.2049	-0.238 to 0.0445	No
Hand D & S vs Hand SPE[A]	0.0420	-0.288 to -0.00546	Yes
CPU SPE vs Hand SPE	0.6806	-0.191 to 0.0912	No

There is a statistical mean difference observed between the groups listed in Tables 7-8 that have the same letter notation, when $\alpha = 0.05$, because these intervals do not contain 0. There is a statistical group mean difference between using the Analyst generated R-values compared to the by hand R-values for meth. Table 9 lists the 95% confidence interval and multiple comparison

mean values that were generated using GraphPad Prism software for amphetamine. Table 10 lists the p-values for the pairwise comparisons when $\alpha = 0.05$ for amphetamine. A result is significant if $p \leq \alpha$.

Table 9: Multiple comparisons 95% confidence interval (CI) values for amphetamine. The same descriptions used in Table 7 were used.

Method	Lower 95% CI	Upper 95% CI
CPU SPE	0.58	1.37
Hand SPE	0.54	1.49
CPU D & S	0.66	0.86
Hand D & S	0.64	1.03

Table 10: Amphetamine Pairwise comparisons p-value & 95% confidence intervals. The same descriptions used in Table 8 were used.

Method Pair	Adjusted p-value	95% CI	Significant?
CPU D & S vs Hand D & S	0.8761	-0.424 to 0.264	No
CPU D & S vs CPU SPE	0.2474	-0.564 to 0.124	No
CPU D & S vs Hand SPE	0.1630	-0.597 to 0.0903	No
Hand D & S vs CPU SPE	0.5852	-0.484 to 0.204	No
Hand D & S vs Hand SPE	0.4226	-0.517 to 0.170	No
CPU SPE vs Hand SPE	0.9888	-0.377 to 0.310	No

The 95% confidence intervals for each separation methods for amphetamine overlap each other, leading to the assumption there is no significant difference between using the Analyst software generated R-values when compared to the by hand R-values for amphetamine. From the pairwise comparison table above, no method pair that is listed is significant because $p \geq \alpha$, when $\alpha = 0.05$.

5.1.2. Pre-Column Derivatization Method Discussion

At this point in time, the extracted samples were not being derivatized because the ion ratios of the derivatized samples for both meth and amphetamine were like the un-derivatized sample ion ratios for either drug. With the inability to derivatize the extracted samples, it was not possible to achieve baseline separation of the enantiomers for with this chiral separation method. Tests were conducted to determine if the urine samples were eluting off the Trace N SPE columns or not. By analyzing the extracted un-derivatized samples and comparing the chromatograms with expected peak retention times gathered from previous testing, it was noted that d-meth and amphetamine were being detected by the instrument, as expected, as shown in Figures 13 and 14.

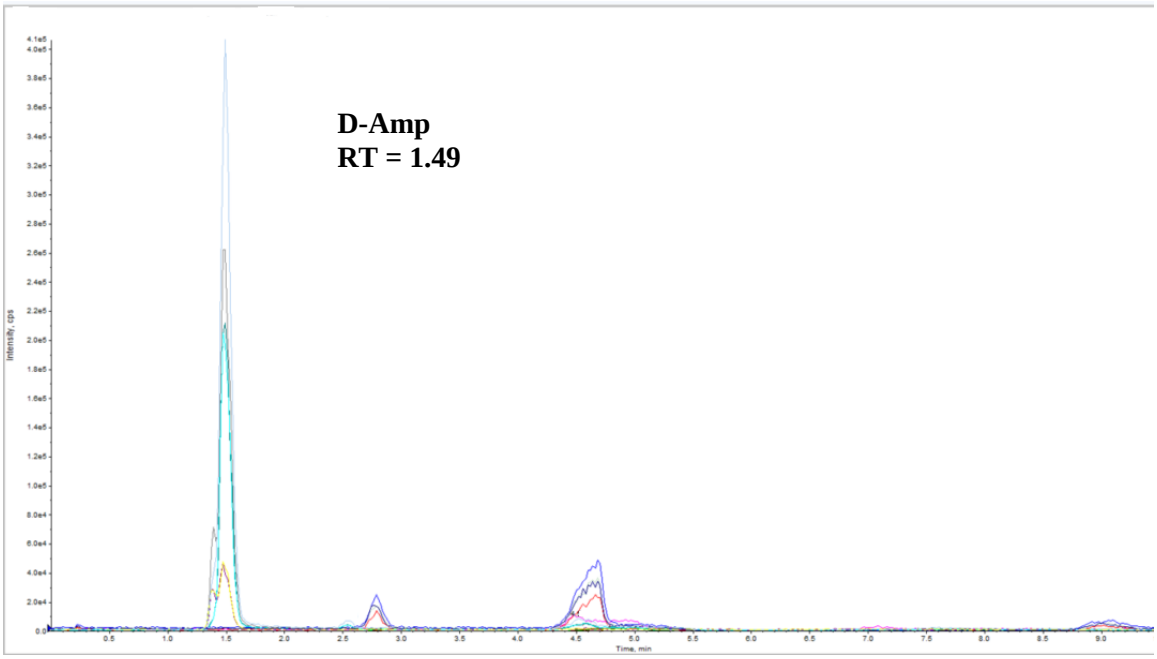

Figure 13: Non-derivatized d-amphetamine

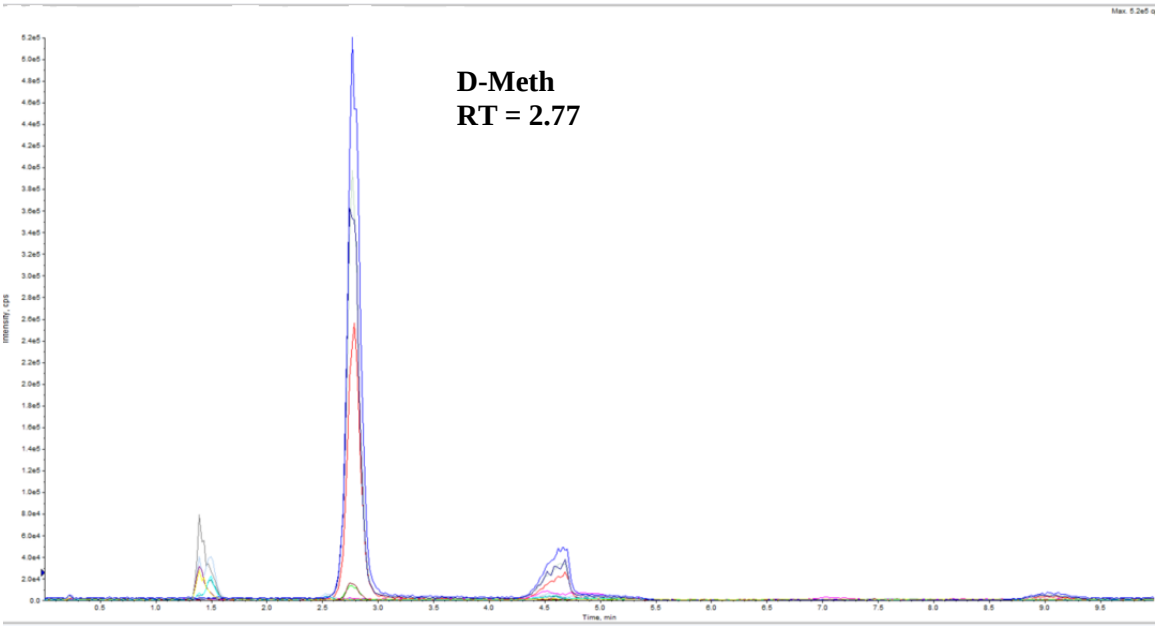

Figure 14: Non-derivatized d-methamphetamine

It could be that the derivatization process that was used was not the correct method for a Restek Raptor Biphenyl achiral column, which is why the derivatized samples were unable to be detected due to the chiral derivatization reagent is not binding to the stationary phase of the column very tightly. The small ion ratios observed for the derivatized samples could also be due to a degraded column, therefore the derivatized analytes are not sticking to the column very tightly to allow for sufficient detection by the instrument.

5.2. Validation

Since the five anonymized urine samples were previously tested for the presence of meth and amphetamine, a multi-point calibration curve was not used at this time. A water sample was injected between each urine sample to assess potential carryover. No added meth or amphetamine was observed in each injected urine or water sample, indicating that no carryover between samples occurred. Before using either extraction scheme for the chiral column separation method, a complete validation study according to SWGTOX guidelines will need to be conducted.[38] A pre-column derivatization method using Marfey's chiral derivatization reagent in urine will need to be developed and validated at a later time so that a comparison study can be conducted between the chiral column and an achiral column pre-column derivatization method to determine which method is more efficient at the enantiomeric separation of meth and amphetamine.

5.3. Study Benefits

One of the major benefits of the study is that it is possible to qualitatively separate the d- and l-enantiomers of meth and amphetamine in urine in-house, rather than sending these positive samples out to an off-site for the separation testing to be conducted. The SPE and D & S separation methods are relatively simple and did not require any specialized chemicals or reagents. While this study focused on a clinical application, the chiral column separation methods can be easily adapted for antemortem settings.

Methamphetamine use in the United States is on the rise. This research provides an example of a clinical application of the qualitative detection and separation of d- and l-enantiomers of meth and amphetamine in positively confirmed meth and/or amphetamine urine samples. The analysis of the type of meth and amphetamine in a sample can potentially provide information about the type of production method used, the identity of the starting materials used for a specific method, and if an individual is compliant with a prescribed drug therapy or not.

5.4. Limitations

While the positive results can imply the type of production method used to produce meth and/or amphetamine, it is not possible to know exactly where an individual acquired the drug. It is possible that the individual in question is legally prescribed Vicks VapoInhalaer™ to clear a stuffy nose.

Another limitation was that the development of a pre-column derivatization method with Marfey's reagent in urine was not completed. Since it was not possible to detect a difference between the extracted derivatized and un-derivatized samples ion ratios, it was not possible to do a complete comparison study between the chiral column separation methods and the pre-column derivatization method.

The SPE extraction and derivatization steps for the pre-column derivatization separation method were very time-consuming when combined, averaging 5.5-6 hours. A forensic and/or clinical lab may wish to shorten the overall analysis time by potentially pairing the derivatization with a liquid-liquid extraction method, which may reduce overall bench time and possibly achieve detection of the derivatized analytes.

Due to time constraints, a complete validation study was not conducted for either chiral separation method, as per SWGTOX guidelines. The validation process would include carryover, interference studies, precision, and limit of detection and quantitation studies.[38]

5.5. Future Research

This study offers several opportunities for additional research. First, the author was unable to get the current derivatization process on the extracted urine samples to work, so was unable to detect derivatized samples on the instrument. A different derivatization process could be utilized, possibly paired with a different extraction method, or a different achiral column, could be analyzed in future research.

Second, an extensive qualitative analysis should be explored for the chiral column separation methods. Only 5 unknown urine samples from one sample batch, provided by the OSU-CLS lab, were tested over 3 days. More samples should be tested to determine if the overall efficiency of the SPE vs D & S separation methods supports or refutes the present study.

Finally, different populations of meth and/or amphetamine users could be investigated. This study focused on the application of clinical samples for drug therapy compliance, but antemortem and postmortem samples could also be analyzed. Examining positively confirmed urine samples from other patient clinics may also be beneficial. Different clinics in Oklahoma may yield different compliance results, which could indicate that a person claiming to be taking a legal form of meth or amphetamine, is really taking the illegal form of either drug, or some variation

5.6. Conclusion

In conclusion, this study presents an option for forensic and clinical toxicology labs wishing to separate d- and l-enantiomers of meth and amphetamine in urine in-house without having to send these positively confirmed samples to an off-site lab for the testing to be done. It is important to note that any lab should consider the overall cost for materials, time, and effort, in addition to the analysis time length, when deciding which chiral separation method to employ.

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